**Genetically Redirected T Lymphocytes for Adoptive Immunotherapy of Solid Tumors.**

Leuci V, Mesiano G, Gammaitoni L, Aglietta M, Sangiolo D.
Genetically Redirected T Lymphocytes for Adoptive Immunotherapy of Solid Tumors.
CURRENT GENE THERAPY (2014) Feb;14

# Genetically redirected T lymphocytes for adoptive immunotherapy of solid tumors

Leuci V[ab], Mesiano G. [ab], Gammaitoni L.[a]., Aglietta M. [ab] and Sangiolo D[ab].

[a] Laboratory of Experimental Cell Therapy, Medical Oncology, Fondazione del Piemonte per l'Oncologia, Institute for Cancer Research and Treatment, 10060 Candiolo (Torino) Italy,

[b] Department of Oncology, University of Torino Medical School, 10060 Candiolo (Torino) Italy,

**Research Support**:

This work was supported in part by grants from "Progetti di Ricerca Rete Oncologia Piemonte-Valle d'Aosta," "Associazione Italiana Ricerca sul Cancro, AIRC I.G. grant. N. 11515, "Associazione Italiana Ricerca sul Cancro–AIRC 5X1000" . The fellowships of L.Gi and M.T., are sponsored by MIUR (University of Turin) and the fellowship of G.M., is sponsored by "Associazione Italiana Ricerca sul Cancro–AIRC I.G. grant. N. 11515.

**Correspondence should be addressed to:**

Dario Sangiolo, MD, PhD.

Laboratory of Cell Therapy, Institute for Cancer Research and Treatment

Provinciale 142 - 10060 Candiolo, Torino Italy.

Phone: +390119933503

Fax: +390119933522

e-mail: dario.sangiolo@ircc.it

**Abstract**

Genetically engineering of T lymphocytes to confer new antitumor specificities is a fascinating approach that may help the successful clinical translation of adoptive immunotherapy strategies. The recognition of tumor-specific antigens may be obtained inducing the membrane expression of transgene encoded antitumor T cell receptors (TCR) or chimeric antigen receptors (CAR). Few but very informative clinical trials with TCR or CAR redirected T lymphocytes have been attempted in the last years, reporting important clinical results along with disappointing failures and important warnings.

In this work we will focus on TCR and CAR redirected T lymphocytes as adoptive immunotherapy for solid tumors. We will review the main topics of these strategies from the angle of clinical applications, discussing the main issues emerged from early clinical trials and their impact on next study designs.

**Adoptive cell therapy for solid tumors.**

Adoptive T cell therapy (ACT) for solid tumors has been a long-time promise in the field of cancer immunotherapy. In the past decades the expectations supported by preclinical studies encountered important limitations in their clinical translation and could not match the relevant results obtained in the field of hematologic malignancies. Overall the main problems were given by difficulties in obtaining clinical relevant numbers of anti-tumor T cells. This issue was partially addressed by recent successes with the infusion of shortly *ex vivo* expanded tumor infiltrating lymphocytes (TIL) in patients with metastatic melanoma (1, 2). Even if very important results with partial and complete remissions were reported, this approach has been so far limited to the setting of metastatic melanoma (1). Several strategies have attempted to select and *ex vivo* expand anti-tumor T lymphocytes in other tumor settings without significant success. The positive results of TIL strategy confirmed however the great potentialities of T cells in cancer therapy, the importance of selecting true tumor antigen-specificity and revitalized research efforts to improve and extend ACT approaches to other solid tumors.

*Genetically redirected T cells*

A very intriguing research branch is exploring the possibility to genetically engineer conventional T cells redirecting their specificity against precise tumor targets. Transforming conventional T cells into tumor-specific lymphocytes would address the limited *ex vivo* expansion capacity of circulating spontaneous tumor-specific T cell precursors.

Progresses in gene-transfer technology have made possible to impart precise and functionally active antigen specificity into conventional T cells. The two main approaches in this direction include the transfer of genes encoding for either a tumor-antigen specific T cell receptor (TCR) or an antibody-based chimeric receptor (CAR).

In this review we will focus on TCR or CAR redirected T cells as treatments for solid tumors. We will approach the main issues of these strategies from the angle of clinical applications, especially reviewing their impact in the initial clinical experiences.

## TCR and CAR redirected T lymphocytes.

### Transgenic TCRs

TCR-transfer strategies require transferring of genetic material encoding for α and β TCR chains with a given specificity for a tumor associated antigens (TAA) presented on cell surface by HLA molecules. The most predominant HLA class-I is HLA-A2, present in ~50% of Caucasians. Consequently, most TCR gene transfer studies have focused on TCR recognizing HLA-A2/peptide complexes. New encoded α/β chains may form undesired dimers with the endogenous TCR chains lowering the expression and the consequent therapeutic power of the intended antitumor transgenic TCR. Furthermore, the new mixed α/β dimers can give rise to unintended recognition of different antigens with potential risks of autoreactivity and toxicities (3, 4). Promising strategies are currently under investigation at preclinical level to minimize the risk for mixed TCR α/β chains. They include modifications of the TCR protein structure to impair the mixed-dimer formation (5-7), knocking down of endogenous TCR chains (8, 9), construction of single-chain antitumor TCRs (10) and introduction of TCRs into γδ T cells(11).

In TCR mediated responses a crucial issue is its affinity for a given tumor antigen. The spontaneous affinity of tumor antigen-specific T cells may usually be low, mostly due to the natural central tolerance toward self-antigens. Strategies under investigation include the isolation of antitumor T cell clones from xenogenic HLA-A2 -transgenic mice (12), definition of gender restricted antigens like in prostate or ovarian cancer and isolation of high affinity TCRs from the allo-HLA repertoire (13, 14). Affinity of antitumor TCR may be also artificially enhanced by genetic approaches such as phage display libraries, or alternatively by improving the steric behavior of the TCR (15-18). It is important to note however that, relatively to TCR affinity, the "more" may not automatically be the "better". Artificially induced maturation of TCR affinity may eventually impair antitumor T cell function. High affinity TCRs realize strong and long-lasting interactions with the presented antigens and may not be properly triggered by tumor antigens that, being at lower concentrations, would require serial multiple TCR triggering (19-21). Secondly, high affinity antitumor TCR may give rise to unpredictable side effects based on cross-reactivity events (22).

**Transgenic CARs**

CARs are chimeric immunoglobulin–TCR molecules (23) derived from transgenes encoding for a single chain variable fragment (scFv) originated from an antibody capable of recognizing a tumor associated antigen. The scFv is fused into a chimeric receptor with TCR-derived signaling domains with consequent T cell activation, upon engagement with the specific tumor antigen on the cell surface, and inducing target cell death using the same mechanisms as ordinary T cells (24, 25).

Current CARs are grouped schematically into three generations with increasing costimulatory activity. First-generation CARs were conjugated with TCR-CD3 zeta chain alone, lacking the intracellular signaling domains of other costimulatory molecules, with consequent suboptimal potential of proliferation activity and cytokine production, eventually affecting a prolonged T-cell expansion and sustained antitumor responses *in vivo*. This result depends on the fact that signal through the TCR-CD3 zeta chain alone is insufficient for priming resting T cells (26). "Second-generation" CARs contain costimulatory signaling domains derived from T cell costimulatory molecules such as CD28, the most commonly used in CAR construction. Besides CD28, other costimulatory molecules such as 4-1BB (CD137), OX40 (CD134), ICOS and CD27, may be included with important roles in regulating T-cell proliferation, survival, and antitumor functions (27-34). The third-generation CARs has been constructed consisting of a CD28, CD3ζ, OX40 or 4-1BB signaling region and have been showed improved signaling capacities compared to the second-generation CARs (35, 36). Besides signaling domain, it is important to consider the spacer/hinge and transmembrane (TM) domains for the final stability and performance ability of CARs. The spacer/hinge region between the antigen-binding and TM domains mediates CAR flexibility and is important for ensuring the suitable positioning of the binding domain during scFv-antigen interactions (37, 38); the TM domain has significant effects on the cell surface expression of CARs. Various TM domains have been used in CAR constructs, such as H2-Kb (39), CD3z (40), FcεRIγ (41), CD4 (33), CD7 (42), CD8 (29) and CD28 (32).

**Functional characteristics of transgenic TCRs and CARs**

Both TCRs and CARs are constructed with the aim of targeting TAA that should ideally be expressed uniquely and specifically by cancer cells.

The main differences between the two strategies regard the typology and modality of target recognition and potential for undesired effects.

Transgenic TCRs are capable to recognize both extracellular and intracellular antigens that need to be processed and presented conjugated with HLA molecules. This amplifies, at least in theory, the range of potential target molecules but the patients who might benefit from this approach might be restricted regarding their HLA haplotype (43). It is of note however that tumor cells have

a tendency to down-regulate HLA class I expression during tumor progression and metastasis formation, which could affect the efficacy TCR-engineered lymphocytes (44).
In spite of the fairly impossibility to find antigens expressed uniquely on tumors and not in normal tissues, several candidate antigens have been identified that are either aberrantly expressed by tumors, such as antigens expressed during the development (e.g. cancer-testis antigens such as MAGE or NY-ESO-1) or overexpressed in tumors compared with normal tissues (such as Her-2/neu or EpCAM) (45, 46).

Differently from TCRs, CAR molecules do not require antigen processing and HLA presentation, recognizing intact target molecules expressed on cell surface. The approach can consequently be applied to all patients regardless their HLA-haplotype but limitation is the incapacity to target intracellular antigens. Even if the range of potential target molecules may in theory appear more limited for CARs compared to TCRs, it is in practice compensated by the easier generation of new antigen-specificities for CARs, depending on availability of new antibodies, compared to the more difficult task of identifying new TCR sequences. CARs present stable membrane expression with intense and sustained intracellular signal transduction that could be enhanced by costimulatory molecules (47). Transgene TCRs may have variations in expression levels according to potential mispairing events with endogenous $\alpha/\beta$ chains (48). The possible development of autoimmunity reactions against transgene products is higher for CAR components, especially if derived from heterologous species (49). Undesired toxicities based on off-target recognitions may happen and have been reported with both strategies (see paragraph below on clinical trials) (50, 51). For TCR receptors new unintended specificities may derive from mispairing with endogenous chains or as consequence of artificial enhancement of affinity (3, 4, 22). Furthermore both TCRs and CARs may mediate reactions against normal tissues that present low expressions of a given antigen (52, 53).

*Gene transfer systems*

Both TCR and CAR based immunotherapy approaches require transferring of genetic material into primary human T cells. Overall the gene transfer can be obtained with viral or non-viral methods. Advances in retroviral and lentiviral DNA transfer have allowed the field to develop rapidly and so far most preclinical and clinical studies have used these two types of vectors to transfer TCR (52, 54-56) and CAR (57-59) genes into T lymphocytes. High levels of transduction efficiency has been achieved by pre-activation of T cells using different systems: agonistic anti-CD3 antibody (OKT-3), CD3/CD28 magnetic beads and artificial antigen presenting cells (APCs), nevertheless pre-activation might impair half-life, immune competence and repertoire of T cells (60). In contrast to retroviral vectors, lentiviral constructs can transduce non-dividing cells, without requiring intense pre-stimulation (61) and can deliver high size transgenes (44-47). Risk

of insertional mutagenesis and malignant transformation using lentiviral and retroviral vectors for TCR and CAR gene transfer is considered very low for fully mature lymphocytes and the safety appears high as confirmed by recent long term follow up reports (62-64). Remaining within viral-based transfer methods it is worth to mention that promising preclinical data exists with Foamy virus vectors, presenting favorable integration and safety features (65). Similarly, adenovirus based vectors may be considered, providing high efficiency when permanent integration is not necessary (66).

Non-viral gene transfer of TCR and CAR using a non-integrating plasmid or *in vitro* transcribed mRNA are currently explored alternatives characterized by lower manufacturing cost and short-term transgene expression, with consequent increased safety but also concerns for a decreased effectiveness of these approaches (31, 67-69). Additional novel approaches worth to note are based on transposon technology, such as PiggyBac (70) or Sleeping Beauty (71, 72), where large sequences with persistent high level transgene expression may become possible.

A schematic summary of the main features of TCR and CAR engineered T lymphocytes is reported in figure 1.

**Clinical translation.**

In the last decade several strategies based on genetically-redirected T cells have entered experimental clinical trials. Even if the majority of experiences are available in the setting of hematologic malignancies, a certain number of informative trials attempted adoptive immunotherapy strategies for solid tumors using either TCR or CAR engineered lymphocytes (Table 1). We will review how the main issues connected to gene-modified T cells impacted the challenging clinical setting of solid tumors in selected trials. In particular we will review and discuss topics related to **1)** clinical response, **2)** *in vivo* T cell persistence, **3)** conditioning lymphodepletion, **4)** toxicity.

***1)*** *Clinical response*. The biologic effect on tumors and improvement of clinical conditions are the obvious ultimate goals of every therapeutic intervention. In the case of ACT for solid tumors, besides objective *clinical response,* also *immunologic responses* and impact on *patient biology* may be very informative. The number of clinical relevant responses registered in advanced solid tumors is still quantitatively limited, not superior to 15-20%. These few events are however extremely important and their interpretation should go beyond their numeric quantification, considering the challenging settings and their prospective value. The settings where TCR-engineered T lymphocytes provided so far the best clinical results are metastatic melanoma and synovial sarcoma (52, 54, 56). Against metastatic melanoma the first clinical results were reported by Morgan et al. that obtained 2 durable complete remissions by ACT with autologous T cells engineered with anti MART-1 TCR (56). Similarly, Johnson and colleagues reported 8 PR

and 1 CR out of 36 patients following ACT with T lymphocytes engineered with TCR recognizing epitopes from MART-1 and GP-100 (52). A different target was successfully exploited by Robbins that, more recently, obtained 6 objective responses, 2 of which durable CR, by the infusion of NY-ESO-specific autologous T cells (54). Within this same trial, NY-ESO-specific T cells were shown for the first time to successfully target metastatic synovial sarcoma reporting 4 clinical relevant responses out of 6 treated patients (54). All patients in these trials were similarly pre-treated with a conditioning lymphodepleting treatment (see paragraph below), based on cyclophosphamide and fludarabine, and the persistence of detectable tumor-specific lymphocytes seemed a common feature that favorably associated with the clinical response.

CAR based strategies have been tested against several types of solid tumors but objective responses were registered only in the setting of neuroblastoma (57). From 2007 three clinical studies were conducted with CAR-engineered T cells against either GD2 or L1 neuroblastoma target molecules (57, 73, 74). Limited but clinically relevant responses were reported, with 3/11 patients with advanced disease achieving CR with anti-GD2 T lymphocytes and 1 PR with anti-L1 T cells. Retroviral vectors were used in all these studies to transfer the TAA-specific CAR and none of the patients received preemptive lymphodepletion treatments. An interesting issue that emerged was the long-term persistence obtained by the strategy of engineering EBV-specific T lymphocytes, likely due to additional costiimulatory signals received by T cells upon infusion.

Both TCR and CAR engineered T lymphocytes were used in other solid tumor settings without obtaining objective responses. Trials were conducted in renal cell carcinoma (CAR vs CAIX) (75, 76), Colorectal cancer (TCR vs CEA and CAR vs ERB-2) (49, 53), ovarian cancer (CAR vs folate receptor) (77). Even if objective responses were almost totally absent, these studies provide informative data especially regarding safety concerns (see dedicated paragraph below).

Besides evaluation of conventional objective response of tumor lesions and clinical benefit, ACT strategies would require the development and application of dedicated assays to assess the immunologic response and biological properties of the infused cell product. The majority of these additional evaluations rely on flow-cytometry assays researching for determinate molecules of tumor-specific lymphocytes. These assays may detect the idiotype of a given scFv incorporated in a CAR or alternatively MHC class I multimers specific for a known TCR (78). Furthermore, PCR based assay may be used to detect molecular components included in the transgenic receptor (50, 79). Caveats related to these analyses are linked to the sensitivity threshold of flow-cytometry, requiring tumor-specific T cells being at least 0.5% of total circulating cells to be detected, and the risk of CAR/TCR internalization upon T cell activation.

PCR based techniques may significantly increase the sensitivity threshold to detect and quantify engineered T cells; molecular analyses, unlike flow-cytometry, do not have however the power to provide crucial phenotype information to appreciate different T cell subsets and speculate on their

functional capacity. Additional insights on T cell functions and immunologic response could be derived by evaluating the production of specific cytokines like TNFα, IFNγ, IL2, IL 4, IL 10 or degranulation levels (80-82). Finally, it is important to consider that all the potential assays to evaluate the quantity and quality of T cell products are almost always performed on peripheral blood samples. While peripheral blood is obviously the easiest and more accessible way, the most informative data on lymphocytes distribution would ideally require direct biopsies at involved tumor sites.

Even pathologic modifications of patient biology, from harmfulness vitiligo to more intense toxic effects, may be indirect insights on the biologic activity of T cell product (83) (see dedicated paragraph below). Overall, definition of strategies to better evaluate and track the quantity, phenotype and functionality of adoptively infused T cells is an ongoing and crucial topic that needs to be developed in parallel to the new expanding ACT approaches.

**2)** *T cell persistence.*

Persistence of infused T lymphocytes and provision of immunologic memory is a crucial point for the successful clinical application of strategies with gene-modified T cells. Low persistence, proliferation exhaustion, immune responses against engineered T cells and decreased transgene expression are important issues that likely contributed to limit the first clinical studies.

The most informative data regarding persistence of TCR-engineered T cells may be deducted from clinical studies against melanoma (2, 49, 52, 54). A long persistence of infused T cells was reported even if the relative low number of enrolled patients not always allowed a clear correlation with the observed clinical response. Interestingly all these studies included a preparative lymphodepleting treatment and IL2 infusion that may have helped the homeostatic proliferation of the infused lymphocytes. Notably it was evident how the time-length of *ex vivo* culture and manipulation had great impact on persistence and quality of infused T cells. Johnson et al. found that only lymphocytes that had been cultured for a shorter time (8-9 days) had longer *in vivo* persistence, apparently associated with objective responses, compared to lymphocytes cultured for longer time length (19 days) (52). Furthermore, short-time cultured T cells were able to revert *in vivo* to a CD45RA+CD45RO- phenotype; this could have been either a real phenotype conversion or due to residual naïve T cells, present at the infusion, that subsequently expanded *in vivo*. In the setting of colorectal cancer engineered lymphocytes with an anti-CEA TCR were documented to persist up to 1 month after the infusion and the low number of patients did not allow any correlation with objective responses (53).

CAR based strategies for solid tumors reported low persistence of infused T cells in the earlier studies against renal cell carcinoma and ovarian cancer, associated with absence of objective responses (75-77). Interesting and promising data may be derived from trials against neuroblastoma using CAR-engineered lymphocytes against GD2 molecule (57, 73). The most intriguing aspect was that a long T cell persistence, associated with clinical response, was reported engineering EBV-specific lymphocytes (84). The underlying idea is that EBV specificity may provide CAR-engineered lymphocytes with a more complete and sustained costimulation upon *in vivo* reinfusion. These data were obtained without preemptive lymphodepletion conditioning and support the idea that quality is probably more important than quantity with relatively low numbers of CAR-engineered lymphocytes capable of relevant results if provided of the correct costimulatory signals. Furthermore the longer persistence of EBV-specific engineered lymphocytes was obtained with first-generation CAR, opening positive expectations for strategies employing second or third generation CARs (73).

Even if not included in the main focus of this review, it is important to refer the initial clinical successes in this direction reported in the setting of B cell malignancies. Relevant clinical responses, significant *in vivo* expansion and persistence of anti-leukemic lymphocytes were obtained with the infusion of T cells engineered with CD19-specific CAR receptor, associated with costimulatory elements given by either CD137-TCRζ or CD28/CD3ζ domains (50, 85).

Furthermore, preclinical data sustain the hypothesis that CD28/CD3ζ costimulation may help CAR-modified T cells to overcome immunosuppressive signals from tumor microenvironment mediated by TGF-beta (86).

Other factors that in theory may impact the persistence of infused engineered T cells may be differences between patient reconstitution of lymphocyte subsets such as T regulatory cells, differences linked to type and stage of tumors and host immune responses against transgene components (87). Strategies to limit the presence and influence of tumor-protective T regulatory cells are currently explored, including lymphodepleting treatments used in these trials, but a definitive conclusion on this issue has not been reached (88, 89). Clinical settings with high tumor burden may provide larger negative conditioning microenvironment but also determine a potential sequestration of infused T cells with apparent lower persistence compared to lower bulky diseases (90). Host immune responses against transgene have been detected in various trials with gene-modified lymphocytes against hematologic malignancies (91). Similar problems should obviously be taken into account also in solid tumor settings. The majority of patients treated with CAR-engineered T cells against CAIX for renal cell carcinoma presented humoral and/or cellular anti–CAIX-CAR T-cell immune responses associated with a limited peripheral persistence of infused T cells (76). Humoral immune responses were anti-idiotypic and capable of impairing the functions of CAR–modified T-cells. Cellular anti–CAIX-CAR immune responses were addressed

against the complementarity-determining and framework regions of the CAR variable domains (52, 75).

As intriguing future prospective, an interesting possibility to enhance the persistence and clinical potency of TCR/CAR redirected lymphocytes could derive by exploiting the "vaccine effect" recently reported with T lymphocytes engineered to express tumor-specific antigens (57, 92).

**3)** *Lymphodepletion.*

Lymphodepleting conditioning therapy seems to positively impact on the efficacy of ACT, confirmed at both preclinical and clinical level (88, 93). It has been demonstrated to increase the homeostatic proliferation of infused T cells, favoring the development of naïve and memory subsets and increasing effectors functional activity (94-96). The exact mechanism underlying the beneficial effect of lymphodepletion is still not completely defined. It is thought to provide "homeostatic proliferation space" to the incoming cells(97), deplete inhibitory T-regs subsets and other lymphocytes that may compete for cytokines uptake, and favor the production of homeostatic cytokines in response to inflammation by chemo or radiotherapy.

Overall clinical objective responses seem to be more present in those studies that included a lymphodepletion before the infusion of engineered T cells. The only exception may be found in the trial based on CAR-engineered T cells against neuroblastoma molecule GD2. Important clinical responses were obtained (see dedicated paragraph above) without any preceding lymphodepleting treatment, but it has to be remarked that in this case anti-tumor lymphocytes were CAR-engineered EBV specific T cells. This strategy aims to obtain antitumor effectors that may encounter additional and adequate co-stimulation and homeostatic signals upon *in vivo* infusion (73). Other studies that did not include conditioning lymphodepleting regimens did not register significant or persistent objective responses in the same neuroblastoma settings or against other solid tumors (75-77).

It is currently not defined which could be the best conditioning regimen and if only chemotherapy or chemo-radiotherapy combination should be used. Some regimens have been reported to preferentially induce immunogenic cell death (98, 99).

Combination of fludarabine and cyclophosphamide is the base of most used lymphodepleting regimens. It was described to increase the response rate of TIL infusion for melanoma patients from about 50% to about 70%. Patients exhibited the expected hematologic toxicities associated with the cyclophosphamide, fludarabine, and total-body irradiation preparative regimens.

An intriguing future prospective may be engineering the appropriate naïve and memory T cell subsets, capable of targeting truly tumor-specific antigens, avoiding or reducing the requirement of lymphodepleting and potentially toxic conditionings of patients.

Similarly, preclinical data indicate the intriguing possibility to target at molecular level the immunosuppressive elements of tumor-associated microenvironment, hypothesis that could eventually obviate or reduce the need for lymphodepleting conditioning treatments. As examples, CD19-specific CAR-modified T lymphocytes, engineered to constitutively secrete IL-12, acquired resistance to inhibition by T regulatory cells and were able to safely eradicate established disease without prior conditioning (93). As other proposed approach, the association of antitumor lymphocytes with CAR-T cells directed against components of tumor stroma seems to significantly enhance the final antitumor effect (100).

**4)** *Toxicity.*

The initial trials with genetically redirected T cells highlighted potential problems linked to expected or unexpected toxicities. Toxicities directly consequent to ACT may be generically described as "on or off-target". The first case is when the event is triggered and directed against the CAR or TCR-cognate targets. The second case may happen when engineered T lymphocytes abnormally recognize molecules other than the original tumor antigen. On-target toxic events, relatively mild, were observed in patients receiving anti MART-1 TCR engineered T lymphocytes, registering episodes of uveitis, skin rash, hearing loss (52, 56). These toxicities could be considered somehow expected as consequence of possible MART-1 expression in tissues other than melanoma (52). Similarly, the expression of CEA molecules in the colic mucosa determined important colitis episodes in patients receiving CAR-modified lymphocytes against CEA as treatment of colorectal cancer (53). Dose-limiting liver toxicity was caused by anti-CAIX CAR-engineered T cells infused as treatment for renal cell carcinoma. In this case, infused lymphocytes were demonstrated to actively infiltrate liver tissue expressing CAIX, and the clinical effect was shown to be prevented by administration of anti-CAIX antibodies at doses that should leave the antigen accessible at tumor sites (76). Potential toxicities may derive from lymphodepleting conditioning regimens, usually based on cyclophosphamide and fludarabine, especially if total body irradiation is associated or if IL2 is infused concomitantly to gene-redirected T lymphocytes (50, 77, 101).

Besides the relatively manageable events, recent trials have also reported the occurrence of fatal on-target off-tumor toxicities. Considering trials for solid tumors, the first episode occurred as consequence of adoptive infusion of CAR-redirected T cells against HER-2 as treatment of colorectal cancer. A severe lung toxicity, cytokine storm and subsequent multi-organ failure occurred shortly after the lymphocyte infusion, likely due to recognizing of low HER-2 expression levels in normal lung cells. It was demonstrated that a marked increase in Interferon-$\gamma$ (IFN-$\gamma$), granulocyte macrophage-colony stimulating factor (GM-CSF), tumor necrosis factor-$\alpha$ (TNF-$\alpha$), interleukin-6 (IL-6) and IL-10 occurred shortly after CAR-engineered T cell infusion. It was found that patient genotype for IL-6, IL10 and TGF-$\beta$1 was associated with higher levels of cytokines

production. This observation may be useful to speculate the mechanistic causes of this event as the specific IL-6 and IL-10 genotypes reported are known to be associated with shock and increased mortality risk in patients with sepsis (102).

None of previous studies involving anti-HER2 antibodies (e.g. Trastuzumab) reported similar fatal events. It is likely that abundant transferring of HER-2 specific T cells can provide a much higher and sustained cytokine production, potentially amplified by further *in vivo* proliferation compared to Trastuzumab (49). The role of pre-infusion conditioning treatment seems less likely to be related to the toxic event, the same research group performed many other trials using the same non-myeloablative regimen, sometimes even with the addition of total body irradiation, without observing any similar side effect.

More insights on cytokine release syndrome may be derived from trials with CAR-modified T cells in the setting of hematologic malignancies. A recent review estimated that about two thirds of patients treated with anti CD19 CAR-T cells reported clinical side effects like fevers, hypotension, fatigue, dyspnea, vascular leak syndrome, tachycardia, liver function impairment and renal failure associated with elevated pro-inflammatory serum cytokines (103).

Cytokines that were mainly monitored were IFN-γ, TNF-α, IL-1β, IL-2, IL-6, IL-7, IL-8, IL-10, IL-12, sIL-2Rα, GM-CSF, their elevation occurred between few days and 3 weeks following infusion of CAR-engineered lymphocytes.

Kochenderfer et al. used a score system based on sequential organ failure assessment (SOFA system) to describe the intensity and severity of cytokine release syndrome; highest scores were associated with elevations in serum levels of IFN-γ and TNF-α (51). Interestingly Brentjens et al. found that intensity of fever and hypotension, as well as levels of cytokine surge, directly correlated with disease burden at the time of CAR-T cells infusion (85). Other elements playing a role in the occurrence and intensity of cytokine release syndrome may be the CAR structure, underlying disease, individual genetic polymorphisms. Clinical experience with cytokine release syndrome following CAR-T cell infusion is still limited but the reported fatal events are indicative of its potential severity. Treatment, when required, is mostly based on corticosteroids and initial evidences support a role for cytokine antagonists like TNF-α inhibitors (e.g. etanercept) and IL-6 receptor-directed therapies (e.g. Tolicizumab) (103, 104).

In some case, especially at initial stages, it could be necessary to differentiate cytokines release syndrome from other clinical conditions like tumor lysis syndrome and sepsis that may also determine elevation of serum cytokines and organ failure. Important alterations of electrolytes (hyperkalemia, hyperphosphatemia and hypocalcaemia), hyperuricemia, microbiologic cultures, specific nucleic acid and antibody assays may help the differential diagnosis, also considering that IFN-γ was reported to be rarely significantly elevated although IL-6 and IL-10 are very high in most patients with sepsis (105, 106).

Fatal events were recently reported also with TCR engineered T lymphocytes. The trial was based on T cells redirected against a MAGE-A3 epitope by enhanced affinity TCR transfer (22). The authors reported that the adverse reactions were linked to myocardial damage by T cell infiltration while no MAGE-A3 expression was found at autopsy. Experimental data revealed that TCR-engineered T lymphocytes, MAGE-A3 specific, were also able to recognize an unrelated epitope, part of the striate muscle protein Titin, providing the first example of clinical off-target off-tumor side effect (22). It should also be considered that the enhanced affinity TCR, used in this trial, may also have concurred in the occurrence of the undesired reaction. Even if this review is focused on solid tumors, it is important to mention that an additional fatal event was reported with the infusion of CAR-engineered T lymphocytes against CD-19. The patient presented with biochemical and clinical signs compatible with tumor lysis syndrome, even if the cause of death is not completely clear and concomitant infectious events may have contributed (107).

The extremely serious events described above induced profound concerns around redirected T cell approaches and highlighted the needs to better investigate issues related to T cell doses, potential inclusion of suicidal switches and specificity checks of affinity matured TCRs/CARs (108).

**Conclusive remarks and future prospective**

Recent data with TCR and CAR modified T lymphocytes demonstrated that gene-transfer redirecting of T cells have the power to potentiate ACT strategies and favor their successful transition from preclinical studies to clinical applications. Besides the initial successes, important issues emerged and are current object of research to further improve and consolidate these approaches. These research topics are either related to TCR and CAR technical improvements or to general issues like safety concerns, optimal antigen selection, gene delivery systems.

The main structural prospective regarding TCR transfer will be the generation of new, tumor selective specificities. In this direction new cloning strategies and the potential exploitation of TCR chain sequences derived from TIL will hopefully promote new results especially against solid tumors (109, 110).

This issue implies limitations due to accessibility of fresh solid tumor samples, with efforts required in this direction to go beyond the exclusive setting of melanoma. As already mentioned, further improvements will likely derive from strategies currently explored at preclinical level, with technical interventions to reduce mispairing events and to modulate TCR affinity for a given tumor antigen. As previously described the artificial maturation of TCR affinity may not automatically result into efficacy improvement and could create safety issues. New predictive preclinical models and/or small scale clinical studies are warranted to careful evaluate these types of interventions.

In regard to CAR-based strategies a main open challenge is definition of the best co-stimulatory pathways for optimal functionality. Even if second and third generations currently seem to have shown higher proliferative and persistence ability, side by side studies should be performed and it is possible that different results may be obtained within different clinical settings.

Intriguing preclinical studies suggest promising strategies, involving combinatorial antigen recognition and physical segregation of costimulatoy elements that could result in tumor-specificity with decreased off-tumor toxicity of CAR-modified T cells (111, 112).

Finally, it is likely that the next future will see multiplied efforts to produce fully humanized CAR molecules, in the attempt of avoiding the risk of deleterious human anti-mouse immune responses (113).

Besides technical improvements of receptors, future perspectives will include the refinement and clinical testing of strategies aiming at limiting or preventing potential toxicities and augmenting the chances of objective antitumor responses. A central role will be played by efforts to identify the right-tumor antigens and the right T cell subsets. Selection of truly tumor-specific targets, like viral antigens in cancers caused by viral infections or mutated molecules due to cancer genome instability, would restrict the immune response to tumor sites preventing abnormal attack of normal tissues. Furthermore a challenging and clinical relevant issue would be to identify and target antigens shared or selectively expressed by putative cancer stem cells, considered responsible of disease relapses and occurrence of chemo-resistance. Similarly, selection of the appropriate T cells will require dedicated attention, involving more naïve and memory subsets or even looking at the recently identified stem cell-like T lymphocytes. Directly linked to these aspects is the consideration that increased tumor-specificity of appropriate lymphocyte subsets may provide efficacy at lower cell doses and could allow to reduce or even avoid pre-conditioning treatments with related potential toxicities.

A parallel prospective research line is the identification and validation of early biomarkers predictive of cytokine storms, with the potential to predict and track side effects of engineered lymphocytes before clinical evidence allowing dose modifications and appropriate therapeutic interventions.

Finally the inclusion of suicide switches into TCR or CAR redirected lymphocytes is a potential countermeasure that could turn-off T cells if undesired clinical side effects occurred. Among the various suicide switches, the most explored and with initial clinical experience available are the HSV-TK and caspase based systems (47, 91, 114, 115).

Even if all together these strategies are likely to produce important safety advancements of gene-redirected immunotherapies, a restricted dose escalation approach appears a general recommendation for future trials involving new artificially-induced antitumor specificities.

Other brilliant strategies are under preclinical investigation to enhance the clinical efficacy of TCR and CAR redirected lymphocytes but cannot be extensively discussed within the framework of this review. It is imaginable that new therapeutic approaches will integrate the current vision of TCR and CAR redirected T lymphocytes; for example new lymphocyte subsets endowed with MHC-unrestricted activity like NK, NKT or Cytokine-induced killer (CIK) cells could be engineered with either TCRs or CARs receptors, conjugating a double MHC-unrestricted and antigen-specific antitumor action on a single cell type susceptible of intense *ex vivo* expansibility.

Finally in the next future it will be important to deal with logistic and economic issues related to these types of strategies. Approaches with redirected T lymphocytes will require testing their efficacy and safety within a more agile system of clinical trials. It will be important to develop studies from pilot small scale, to evaluate new ideas, to larger studies on randomized basis to compare the efficacy with conventional therapies. Costs and complexity of manufacturing engineered T cell products remain an issue. It seems difficult to imagine that numerous Centers and Hospitals could realize their-own cell factory capable to produce engineered T cells for clinical use. It seems reasonable for safety and cost effectiveness to promote and sustain few high-quality cell factories on regional basis, which could produce and provide the required T cell product to the requiring hospital within that region.

Either way, it seems that the clinical application of redirected T cells for anticancer application, is finally gaining the adequate consideration. Even if many issues need to be addressed it is possible and desirable that these approaches will offer new chances and hopes for clinical trials against incurable solid tumors.

References

1. Donia M, Junker N, Ellebaek E, Andersen MH, Straten PT, Svane IM. Characterization and comparison of "Standard" and "Young" tumor infiltrating lymphocytes for adoptive cell therapy at a Danish Translational Research Institution. Scand J Immunol. 2011.

2. Phan GQ, Rosenberg SA. Adoptive cell transfer for patients with metastatic melanoma: the potential and promise of cancer immunotherapy. Cancer Control. 2013;20:289-97.

3. Bendle GM, Linnemann C, Hooijkaas AI, Bies L, de Witte MA, Jorritsma A, et al. Lethal graft-versus-host disease in mouse models of T cell receptor gene therapy. Nat Med. 2010;16:565-70, 1p following 70.

4. van Loenen MM, de Boer R, Amir AL, Hagedoorn RS, Volbeda GL, Willemze R, et al. Mixed T cell receptor dimers harbor potentially harmful neoreactivity. Proc Natl Acad Sci U S A. 2010;107:10972-7.

5. Cohen CJ, Li YF, El-Gamil M, Robbins PF, Rosenberg SA, Morgan RA. Enhanced antitumor activity of T cells engineered to express T-cell receptors with a second disulfide bond. Cancer Res. 2007;67:3898-903.
6. Sommermeyer D, Uckert W. Minimal amino acid exchange in human TCR constant regions fosters improved function of TCR gene-modified T cells. J Immunol. 2010;184:6223-31.
7. Kuball J, Dossett ML, Wolfl M, Ho WY, Voss RH, Fowler C, et al. Facilitating matched pairing and expression of TCR chains introduced into human T cells. Blood. 2007;109:2331-8.
8. Ochi T, Fujiwara H, Okamoto S, An J, Nagai K, Shirakata T, et al. Novel adoptive T-cell immunotherapy using a WT1-specific TCR vector encoding silencers for endogenous TCRs shows marked antileukemia reactivity and safety. Blood. 2011;118:1495-503.
9. Provasi E, Genovese P, Lombardo A, Magnani Z, Liu PQ, Reik A, et al. Editing T cell specificity towards leukemia by zinc finger nucleases and lentiviral gene transfer. Nat Med. 2012;18:807-15.
10. Voss RH, Thomas S, Pfirschke C, Hauptrock B, Klobuch S, Kuball J, et al. Coexpression of the T-cell receptor constant alpha domain triggers tumor reactivity of single-chain TCR-transduced human T cells. Blood. 2010;115:5154-63.
11. van der Veken LT, Hagedoorn RS, van Loenen MM, Willemze R, Falkenburg JH, Heemskerk MH. Alphabeta T-cell receptor engineered gammadelta T cells mediate effective antileukemic reactivity. Cancer Res. 2006;66:3331-7.
12. Lustgarten J, Dominguez AL, Cuadros C. The CD8+ T cell repertoire against Her-2/neu antigens in neu transgenic mice is of low avidity with antitumor activity. Eur J Immunol. 2004;34:752-61.
13. Savage P, Gao L, Vento K, Cowburn P, Man S, Steven N, et al. Use of B cell-bound HLA-A2 class I monomers to generate high-avidity, allo-restricted CTLs against the leukemia-associated protein Wilms tumor antigen. Blood. 2004;103:4613-5.
14. Friedman RS, Spies AG, Kalos M. Identification of naturally processed CD8 T cell epitopes from prostein, a prostate tissue-specific vaccine candidate. Eur J Immunol. 2004;34:1091-101.
15. Chervin AS, Aggen DH, Raseman JM, Kranz DM. Engineering higher affinity T cell receptors using a T cell display system. J Immunol Methods. 2008;339:175-84.
16. Li Y, Moysey R, Molloy PE, Vuidepot AL, Mahon T, Baston E, et al. Directed evolution of human T-cell receptors with picomolar affinities by phage display. Nat Biotechnol. 2005;23:349-54.
17. Robbins PF, Li YF, El-Gamil M, Zhao Y, Wargo JA, Zheng Z, et al. Single and dual amino acid substitutions in TCR CDRs can enhance antigen-specific T cell functions. J Immunol. 2008;180:6116-31.
18. Kuball J, Hauptrock B, Malina V, Antunes E, Voss RH, Wolfl M, et al. Increasing functional avidity of TCR-redirected T cells by removing defined N-glycosylation sites in the TCR constant domain. J Exp Med. 2009;206:463-75.
19. Thomas S, Xue SA, Bangham CR, Jakobsen BK, Morris EC, Stauss HJ. Human T cells expressing affinity-matured TCR display accelerated responses but fail to recognize low density of MHC-peptide antigen. Blood. 2011;118:319-29.

20. Valitutti S, Müller S, Cella M, Padovan E, Lanzavecchia A. Serial triggering of many T-cell receptors by a few peptide-MHC complexes. Nature. 1995;375:148-51.
21. Valitutti S, Müller S, Salio M, Lanzavecchia A. Degradation of T cell receptor (TCR)-CD3-zeta complexes after antigenic stimulation. J Exp Med. 1997;185:1859-64.
22. Linette GP, Stadtmauer EA, Maus MV, Rapoport AP, Levine BL, Emery L, et al. Cardiovascular toxicity and titin cross-reactivity of affinity-enhanced T cells in myeloma and melanoma. Blood. 2013;122:863-71.
23. Gross G, Waks T, Eshhar Z. Expression of immunoglobulin-T-cell receptor chimeric molecules as functional receptors with antibody-type specificity. Proc Natl Acad Sci U S A. 1989;86:10024-8.
24. Dotti G, Savoldo B, Brenner M. Fifteen years of gene therapy based on chimeric antigen receptors: "are we nearly there yet?". Hum Gene Ther. 2009;20:1229-39.
25. Hombach A, Köhler H, Rappl G, Abken H. Human CD4+ T cells lyse target cells via granzyme/perforin upon circumvention of MHC class II restriction by an antibody-like immunoreceptor. J Immunol. 2006;177:5668-75.
26. Brocker T, Karjalainen K. Signals through T cell receptor-zeta chain alone are insufficient to prime resting T lymphocytes. J Exp Med. 1995;181:1653-9.
27. Kowolik CM, Topp MS, Gonzalez S, Pfeiffer T, Olivares S, Gonzalez N, et al. CD28 costimulation provided through a CD19-specific chimeric antigen receptor enhances in vivo persistence and antitumor efficacy of adoptively transferred T cells. Cancer Res. 2006;66:10995-1004.
28. Hombach A, Wieczarkowiecz A, Marquardt T, Heuser C, Usai L, Pohl C, et al. Tumor-specific T cell activation by recombinant immunoreceptors: CD3 zeta signaling and CD28 costimulation are simultaneously required for efficient IL-2 secretion and can be integrated into one combined CD28/CD3 zeta signaling receptor molecule. J Immunol. 2001;167:6123-31.
29. Song DG, Ye Q, Carpenito C, Poussin M, Wang LP, Ji C, et al. In vivo persistence, tumor localization, and antitumor activity of CAR-engineered T cells is enhanced by costimulatory signaling through CD137 (4-1BB). Cancer Res. 2011;71:4617-27.
30. Hombach AA, Abken H. Costimulation by chimeric antigen receptors revisited the T cell antitumor response benefits from combined CD28-OX40 signalling. Int J Cancer. 2011;129:2935-44.
31. Birkholz K, Hombach A, Krug C, Reuter S, Kershaw M, Kämpgen E, et al. Transfer of mRNA encoding recombinant immunoreceptors reprograms CD4+ and CD8+ T cells for use in the adoptive immunotherapy of cancer. Gene Ther. 2009;16:596-604.
32. Song DG, Ye Q, Poussin M, Harms GM, Figini M, Powell DJ. CD27 costimulation augments the survival and antitumor activity of redirected human T cells in vivo. Blood. 2012;119:696-706.
33. Milone MC, Fish JD, Carpenito C, Carroll RG, Binder GK, Teachey D, et al. Chimeric receptors containing CD137 signal transduction domains mediate enhanced survival of T cells and increased antileukemic efficacy in vivo. Mol Ther. 2009;17:1453-64.
34. Tammana S, Huang X, Wong M, Milone MC, Ma L, Levine BL, et al. 4-1BB and CD28 signaling plays a synergistic role in redirecting umbilical cord blood T cells against B-cell malignancies. Hum Gene Ther. 2010;21:75-86.

35. Pulè MA, Straathof KC, Dotti G, Heslop HE, Rooney CM, Brenner MK. A chimeric T cell antigen receptor that augments cytokine release and supports clonal expansion of primary human T cells. Mol Ther. 2005;12:933-41.
36. Hombach AA, Heiders J, Foppe M, Chmielewski M, Abken H. OX40 costimulation by a chimeric antigen receptor abrogates CD28 and IL-2 induced IL-10 secretion by redirected CD4(+) T cells. Oncoimmunology. 2012;1:458-66.
37. Bridgeman JS, Hawkins RE, Hombach AA, Abken H, Gilham DE. Building better chimeric antigen receptors for adoptive T cell therapy. Curr Gene Ther. 2010;10:77-90.
38. Shirasu N, Kuroki M. Functional design of chimeric T-cell antigen receptors for adoptive immunotherapy of cancer: architecture and outcomes. Anticancer Res. 2012;32:2377-83.
39. Church DN, Talbot DC. Survivin in solid tumors: rationale for development of inhibitors. Curr Oncol Rep. 2012;14:120-8.
40. Kahlon KS, Brown C, Cooper LJ, Raubitschek A, Forman SJ, Jensen MC. Specific recognition and killing of glioblastoma multiforme by interleukin 13-zetakine redirected cytolytic T cells. Cancer Res. 2004;64:9160-6.
41. Schambach A, Swaney WP, van der Loo JC. Design and production of retro- and lentiviral vectors for gene expression in hematopoietic cells. Methods Mol Biol. 2009;506:191-205.
42. Sinn PL, Sauter SL, McCray PB. Gene therapy progress and prospects: development of improved lentiviral and retroviral vectors--design, biosafety, and production. Gene Ther. 2005;12:1089-98.
43. Clay TM, Custer MC, Sachs J, Hwu P, Rosenberg SA, Nishimura MI. Efficient transfer of a tumor antigen-reactive TCR to human peripheral blood lymphocytes confers anti-tumor reactivity. J Immunol. 1999;163:507-13.
44. Zhang H, Melamed J, Wei P, Cox K, Frankel W, Bahnson RR, et al. Concordant down-regulation of proto-oncogene PML and major histocompatibility antigen HLA class I expression in high-grade prostate cancer. Cancer Immun. 2003;3:2.
45. Caballero OL, Chen YT. Cancer/testis (CT) antigens: potential targets for immunotherapy. Cancer Sci. 2009;100:2014-21.
46. Old LJ. Cancer vaccines: an overview. Cancer Immun. 2008;8 Suppl 1:1.
47. Stauss HJ, Morris EC. Immunotherapy with gene-modified T cells: limiting side effects provides new challenges. Gene Ther. 2013.
48. Thomas S, Xue SA, Cesco-Gaspere M, San José E, Hart DP, Wong V, et al. Targeting the Wilms tumor antigen 1 by TCR gene transfer: TCR variants improve tetramer binding but not the function of gene modified human T cells. J Immunol. 2007;179:5803-10.
49. Morgan RA, Yang JC, Kitano M, Dudley ME, Laurencot CM, Rosenberg SA. Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. Mol Ther. 2010;18:843-51.
50. Kalos M, Levine BL, Porter DL, Katz S, Grupp SA, Bagg A, et al. T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. Sci Transl Med. 2011;3:95ra73.
51. Kochenderfer JN, Dudley ME, Feldman SA, Wilson WH, Spaner DE, Maric I, et al. B-cell depletion and remissions of malignancy along with cytokine-associated toxicity

in a clinical trial of anti-CD19 chimeric-antigen-receptor-transduced T cells. Blood. 2012;119:2709-20.
52. Johnson LA, Morgan RA, Dudley ME, Cassard L, Yang JC, Hughes MS, et al. Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. Blood. 2009;114:535-46.
53. Parkhurst MR, Yang JC, Langan RC, Dudley ME, Nathan DA, Feldman SA, et al. T cells targeting carcinoembryonic antigen can mediate regression of metastatic colorectal cancer but induce severe transient colitis. Mol Ther. 2011;19:620-6.
54. Robbins PF, Morgan RA, Feldman SA, Yang JC, Sherry RM, Dudley ME, et al. Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. J Clin Oncol. 2011;29:917-24.
55. Varela-Rohena A, Molloy PE, Dunn SM, Li Y, Suhoski MM, Carroll RG, et al. Control of HIV-1 immune escape by CD8 T cells expressing enhanced T-cell receptor. Nat Med. 2008;14:1390-5.
56. Morgan RA, Dudley ME, Wunderlich JR, Hughes MS, Yang JC, Sherry RM, et al. Cancer regression in patients after transfer of genetically engineered lymphocytes. Science. 2006;314:126-9.
57. Pule MA, Savoldo B, Myers GD, Rossig C, Russell HV, Dotti G, et al. Virus-specific T cells engineered to coexpress tumor-specific receptors: persistence and antitumor activity in individuals with neuroblastoma. Nat Med. 2008;14:1264-70.
58. Till BG, Jensen MC, Wang J, Chen EY, Wood BL, Greisman HA, et al. Adoptive immunotherapy for indolent non-Hodgkin lymphoma and mantle cell lymphoma using genetically modified autologous CD20-specific T cells. Blood. 2008;112:2261-71.
59. Jena B, Dotti G, Cooper LJ. Redirecting T-cell specificity by introducing a tumor-specific chimeric antigen receptor. Blood. 2010;116:1035-44.
60. Sauce D, Tonnelier N, Duperrier A, Petracca B, de Carvalho Bittencourt M, Saadi M, et al. Influence of ex vivo expansion and retrovirus-mediated gene transfer on primary T lymphocyte phenotype and functions. J Hematother Stem Cell Res. 2002;11:929-40.
61. Cavalieri S, Cazzaniga S, Geuna M, Magnani Z, Bordignon C, Naldini L, et al. Human T lymphocytes transduced by lentiviral vectors in the absence of TCR activation maintain an intact immune competence. Blood. 2003;102:497-505.
62. Biffi A, Bartolomae CC, Cesana D, Cartier N, Aubourg P, Ranzani M, et al. Lentiviral vector common integration sites in preclinical models and a clinical trial reflect a benign integration bias and not oncogenic selection. Blood. 2011;117:5332-9.
63. Scholler J, Brady TL, Binder-Scholl G, Hwang WT, Plesa G, Hege KM, et al. Decade-long safety and function of retroviral-modified chimeric antigen receptor T cells. Sci Transl Med. 2012;4:132ra53.
64. Bonini C, Grez M, Traversari C, Ciceri F, Marktel S, Ferrari G, et al. Safety of retroviral gene marking with a truncated NGF receptor. Nat Med. 2003;9:367-9.
65. Lindemann D, Rethwilm A. Foamy virus biology and its application for vector development. Viruses. 2011;3:561-85.
66. Perez EE, Wang J, Miller JC, Jouvenot Y, Kim KA, Liu O, et al. Establishment of HIV-1 resistance in CD4+ T cells by genome editing using zinc-finger nucleases. Nat Biotechnol. 2008;26:808-16.

67. Zhao Y, Zheng Z, Cohen CJ, Gattinoni L, Palmer DC, Restifo NP, et al. High-efficiency transfection of primary human and mouse T lymphocytes using RNA electroporation. Mol Ther. 2006;13:151-9.
68. Zhao Y, Moon E, Carpenito C, Paulos CM, Liu X, Brennan AL, et al. Multiple injections of electroporated autologous T cells expressing a chimeric antigen receptor mediate regression of human disseminated tumor. Cancer Res. 2010;70:9053-61.
69. Barrett DM, Zhao Y, Liu X, Jiang S, Carpenito C, Kalos M, et al. Treatment of advanced leukemia in mice with mRNA engineered T cells. Hum Gene Ther. 2011;22:1575-86.
70. Wilson MH, Coates CJ, George AL. PiggyBac transposon-mediated gene transfer in human cells. Mol Ther. 2007;15:139-45.
71. Dupuy AJ, Akagi K, Largaespada DA, Copeland NG, Jenkins NA. Mammalian mutagenesis using a highly mobile somatic Sleeping Beauty transposon system. Nature. 2005;436:221-6.
72. Hackett PB, Largaespada DA, Cooper LJ. A transposon and transposase system for human application. Mol Ther. 2010;18:674-83.
73. Louis CU, Savoldo B, Dotti G, Pule M, Yvon E, Myers GD, et al. Antitumor activity and long-term fate of chimeric antigen receptor-positive T cells in patients with neuroblastoma. Blood. 2011;118:6050-6.
74. Park JR, Digiusto DL, Slovak M, Wright C, Naranjo A, Wagner J, et al. Adoptive transfer of chimeric antigen receptor re-directed cytolytic T lymphocyte clones in patients with neuroblastoma. Mol Ther. 2007;15:825-33.
75. Lamers CH, Langeveld SC, Groot-van Ruijven CM, Debets R, Sleijfer S, Gratama JW. Gene-modified T cells for adoptive immunotherapy of renal cell cancer maintain transgene-specific immune functions in vivo. Cancer Immunol Immunother. 2007;56:1875-83.
76. Lamers CH, Willemsen R, van Elzakker P, van Steenbergen-Langeveld S, Broertjes M, Oosterwijk-Wakka J, et al. Immune responses to transgene and retroviral vector in patients treated with ex vivo-engineered T cells. Blood. 2011;117:72-82.
77. Kershaw MH, Westwood JA, Parker LL, Wang G, Eshhar Z, Mavroukakis SA, et al. A phase I study on adoptive immunotherapy using gene-modified T cells for ovarian cancer. Clin Cancer Res. 2006;12:6106-15.
78. Gratama JW, Kern F. Flow cytometric enumeration of antigen-specific T lymphocytes. Cytometry A. 2004;58:79-86.
79. Duval L, Schmidt H, Kaltoft K, Fode K, Jensen JJ, Sorensen SM, et al. Adoptive transfer of allogeneic cytotoxic T lymphocytes equipped with a HLA-A2 restricted MART-1 T-cell receptor: a phase I trial in metastatic melanoma. Clin Cancer Res. 2006;12:1229-36.
80. Betts MR, Harari A. Phenotype and function of protective T cell immune responses in HIV. Curr Opin HIV AIDS. 2008;3:349-55.
81. Gaucher D, Therrien R, Kettaf N, Angermann BR, Boucher G, Filali-Mouhim A, et al. Yellow fever vaccine induces integrated multilineage and polyfunctional immune responses. J Exp Med. 2008;205:3119-31.
82. Makedonas G, Hutnick N, Haney D, Amick AC, Gardner J, Cosma G, et al. Perforin and IL-2 upregulation define qualitative differences among highly functional virus-specific human CD8 T cells. PLoS Pathog. 2010;6:e1000798.

83. Rapoport AP, Stadtmauer EA, Aqui N, Vogl D, Chew A, Fang HB, et al. Rapid immune recovery and graft-versus-host disease-like engraftment syndrome following adoptive transfer of Costimulated autologous T cells. Clin Cancer Res. 2009;15:4499-507.
84. Louis CU, Straathof K, Bollard CM, Ennamuri S, Gerken C, Lopez TT, et al. Adoptive transfer of EBV-specific T cells results in sustained clinical responses in patients with locoregional nasopharyngeal carcinoma. J Immunother. 2010;33:983-90.
85. Brentjens RJ, Davila ML, Riviere I, Park J, Wang X, Cowell LG, et al. CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia. Sci Transl Med. 2013;5:177ra38.
86. Koehler H, Kofler D, Hombach A, Abken H. CD28 costimulation overcomes transforming growth factor-beta-mediated repression of proliferation of redirected human CD4+ and CD8+ T cells in an antitumor cell attack. Cancer Res. 2007;67:2265-73.
87. Melenhorst JJ, Scheinberg P, Chattopadhyay PK, Gostick E, Ladell K, Roederer M, et al. High avidity myeloid leukemia-associated antigen-specific CD8+ T cells preferentially reside in the bone marrow. Blood. 2009;113:2238-44.
88. Brentjens RJ, Rivière I, Park JH, Davila ML, Wang X, Stefanski J, et al. Safety and persistence of adoptively transferred autologous CD19-targeted T cells in patients with relapsed or chemotherapy refractory B-cell leukemias. Blood. 2011;118:4817-28.
89. Loskog A, Giandomenico V, Rossig C, Pule M, Dotti G, Brenner MK. Addition of the CD28 signaling domain to chimeric T-cell receptors enhances chimeric T-cell resistance to T regulatory cells. Leukemia. 2006;20:1819-28.
90. Fisher DT, Chen Q, Appenheimer MM, Skitzki J, Wang WC, Odunsi K, et al. Hurdles to lymphocyte trafficking in the tumor microenvironment: implications for effective immunotherapy. Immunol Invest. 2006;35:251-77.
91. Berger C, Flowers ME, Warren EH, Riddell SR. Analysis of transgene-specific immune responses that limit the in vivo persistence of adoptively transferred HSV-TK-modified donor T cells after allogeneic hematopoietic cell transplantation. Blood. 2006;107:2294-302.
92. Russo V, Bondanza A, Ciceri F, Bregni M, Bordignon C, Traversari C, et al. A dual role for genetically modified lymphocytes in cancer immunotherapy. Trends Mol Med. 2012;18:193-200.
93. Pegram HJ, Lee JC, Hayman EG, Imperato GH, Tedder TF, Sadelain M, et al. Tumor-targeted T cells modified to secrete IL-12 eradicate systemic tumors without need for prior conditioning. Blood. 2012;119:4133-41.
94. Dudley ME, Wunderlich JR, Robbins PF, Yang JC, Hwu P, Schwartzentruber DJ, et al. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. Science. 2002;298:850-4.
95. Muranski P, Boni A, Wrzesinski C, Citrin DE, Rosenberg SA, Childs R, et al. Increased intensity lymphodepletion and adoptive immunotherapy--how far can we go? Nat Clin Pract Oncol. 2006;3:668-81.
96. Wrzesinski C, Restifo NP. Less is more: lymphodepletion followed by hematopoietic stem cell transplant augments adoptive T-cell-based anti-tumor immunotherapy. Curr Opin Immunol. 2005;17:195-201.

97. Gattinoni L, Finkelstein SE, Klebanoff CA, Antony PA, Palmer DC, Spiess PJ, et al. Removal of homeostatic cytokine sinks by lymphodepletion enhances the efficacy of adoptively transferred tumor-specific CD8+ T cells. J Exp Med. 2005;202:907-12.
98. Machiels JP, Reilly RT, Emens LA, Ercolini AM, Lei RY, Weintraub D, et al. Cyclophosphamide, doxorubicin, and paclitaxel enhance the antitumor immune response of granulocyte/macrophage-colony stimulating factor-secreting whole-cell vaccines in HER-2/neu tolerized mice. Cancer Res. 2001;61:3689-97.
99. Obeid M, Tesniere A, Ghiringhelli F, Fimia GM, Apetoh L, Perfettini JL, et al. Calreticulin exposure dictates the immunogenicity of cancer cell death. Nat Med. 2007;13:54-61.
100. Kakarla S, Chow KKh, Mata M, Shaffer DR, Song XT, Wu MF, et al. Antitumor effects of chimeric receptor engineered human T cells directed to tumor stroma. Mol Ther. 2013;21:1611-20.
101. Gill S, Kalos M. T cell-based gene therapy of cancer. Transl Res. 2013;161:365-79.
102. Cohen CJ, Zhao Y, Zheng Z, Rosenberg SA, Morgan RA. Enhanced antitumor activity of murine-human hybrid T-cell receptor (TCR) in human lymphocytes is associated with improved pairing and TCR/CD3 stability. Cancer Res. 2006;66:8878-86.
103. Xu XJ, Tang YM. Cytokine release syndrome in cancer immunotherapy with chimeric antigen receptor engineered T cells. Cancer Lett. 2013.
104. Grupp SA, Kalos M, Barrett D, Aplenc R, Porter DL, Rheingold SR, et al. Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. N Engl J Med. 2013;368:1509-18.
105. Xu XJ, Tang YM, Song H, Yang SL, Xu WQ, Zhao N, et al. Diagnostic accuracy of a specific cytokine pattern in hemophagocytic lymphohistiocytosis in children. J Pediatr. 2012;160:984-90.e1.
106. Xu XJ, Tang YM, Liao C, Song H, Yang SL, Xu WQ, et al. Inflammatory cytokine measurement quickly discriminates gram-negative from gram-positive bacteremia in pediatric hematology/oncology patients with septic shock. Intensive Care Med. 2013;39:319-26.
107. Brentjens R, Yeh R, Bernal Y, Riviere I, Sadelain M. Treatment of chronic lymphocytic leukemia with genetically targeted autologous T cells: case report of an unforeseen adverse event in a phase I clinical trial. Mol Ther. 2010;18:666-8.
108. Ertl HC, Zaia J, Rosenberg SA, June CH, Dotti G, Kahn J, et al. Considerations for the clinical application of chimeric antigen receptor T cells: observations from a recombinant DNA Advisory Committee Symposium held June 15, 2010. Cancer Res. 2011;71:3175-81.
109. Birkholz K, Hofmann C, Hoyer S, Schulz B, Harrer T, Kämpgen E, et al. A fast and robust method to clone and functionally validate T-cell receptors. J Immunol Methods. 2009;346:45-54.
110. Seitz S, Schneider CK, Malotka J, Nong X, Engel AG, Wekerle H, et al. Reconstitution of paired T cell receptor alpha- and beta-chains from microdissected single cells of human inflammatory tissues. Proc Natl Acad Sci U S A. 2006;103:12057-62.

111. Kloss CC, Condomines M, Cartellieri M, Bachmann M, Sadelain M. Combinatorial antigen recognition with balanced signaling promotes selective tumor eradication by engineered T cells. Nat Biotechnol. 2013;31:71-5.
112. Evripidis Lanitis MP, Alex W. Klattenhoff, Degang Song,Raphael Sandaltzopoulos, Carl H. June, and Daniel J. Powell Jr. Chimeric Antigen Receptor T Cells with Dissociated Signaling Domains Exhibit Focused Antitumor Activity with Reduced Potential for Toxicity *In Vivo*. Cancer Immunology Research. 2013.
113. Lamers CH, Sleijfer S, Vulto AG, Kruit WH, Kliffen M, Debets R, et al. Treatment of metastatic renal cell carcinoma with autologous T-lymphocytes genetically retargeted against carbonic anhydrase IX: first clinical experience. J Clin Oncol. 2006;24:e20-2.
114. Traversari C, Marktel S, Magnani Z, Mangia P, Russo V, Ciceri F, et al. The potential immunogenicity of the TK suicide gene does not prevent full clinical benefit associated with the use of TK-transduced donor lymphocytes in HSCT for hematologic malignancies. Blood. 2007;109:4708-15.
115. Di Stasi A, Tey SK, Dotti G, Fujita Y, Kennedy-Nasser A, Martinez C, et al. Inducible apoptosis as a safety switch for adoptive cell therapy. N Engl J Med. 2011;365:1673-83.

| Disease | Target Antigen | Pre-conditioning/ exogenous cytokines | Transgene Receptor | Outcome | Toxicity | Ref. |
|---|---|---|---|---|---|---|
| Melanoma | MART-1 gp-100 | (CTX-FLU)/IL-2 | TCR | 8 PR 1CR | Skin, eyes, ear | Johnson 2009, Morgan 2006 |
| Synovial sarcoma; melanoma | NY-ESO1 | (CTX-FLU)/IL-2 | TCR | 9 PR 2 CR | None | Robbins 2011 |
| Renal cell sarcoma | CAIX | None | CAR($1^{st}$ generation) | No objective clinical response | Hepatotoxicity | Lamers 2007 Lamers 2011 |
| Colorectal cancer | CEA | (CTX-FLU)/IL-2 | TCR | 1 PR | Transient colitis | Parkhurst 2011 |
| Colorectal cancer | ErbB2 | (CTX-FLU)/IL-2 | CAR($3^{rd}$ generation) | N.A. | Fatal lung toxicity and organ failure. | Morgan 2010 |
| Ovarian cancer | αFR | None/IL2 | CAR($1^{st}$ generation) | None | Due to IL2 | Kershaw 2006 |
| Neuroblastoma | GD2 | None | CAR($1^{st}$ generation) | 3 CR | Pain at tumor site | Pule 2008, Louis 2011 |
| Neuroblastoma | L1 | None | CAR($1^{st}$ generation) | 1 PR | Pneumonitis, cytopenias | Park 2007 |

**Table 1. Summary of selected clinical trials based on the use of TCR and CAR engineered T-cells for treatment of solid tumors**

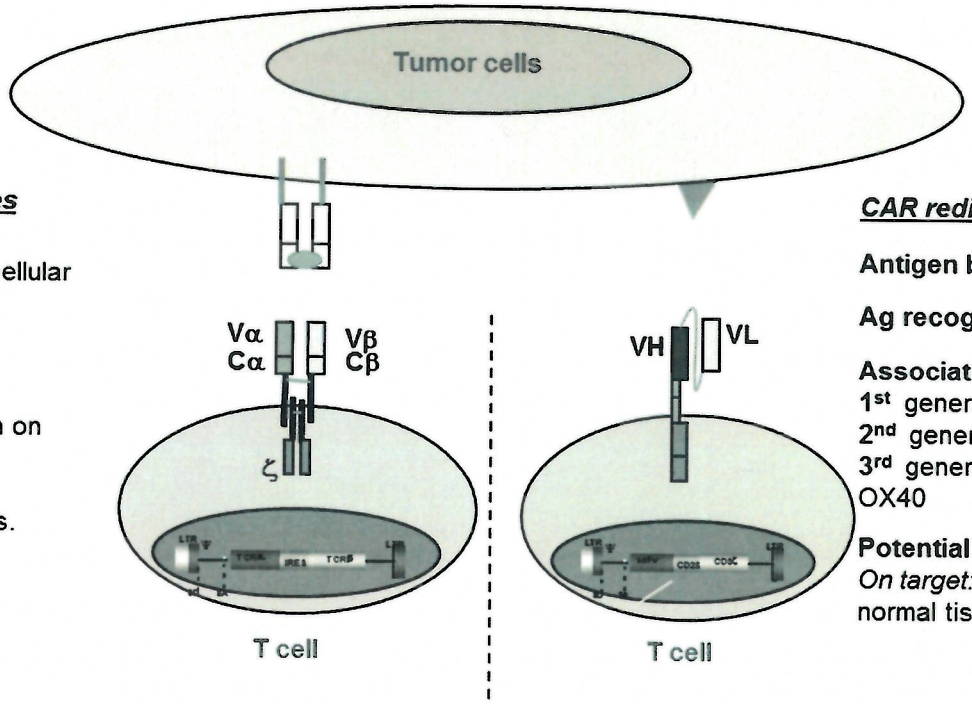
**Abbreviations**: αFR: Alpha folate receptor; CAR: Chimeric Antigen receptor; CAIX: carbonic anhydrase IX; CEA: Carcinoembryonic antigen; CR: Complete response; CTX: Cyclophosphamide (60 mg/kg); FLU: Fludarabine (25 $mg/m^2$); IL2: interleukine 2 ($7{,}2 \times 10^5$ U/kg); GD2: Disialoganglioside2; OR: Objective responses; N.A.: Not available; PR: Partial response; TCR: T-Cell Receptor

***TCR redirected T lymphocytes***

**Antigen binding**:extracellular or intracellular

**Ag recognition:** HLA restricted

**Potential toxicities**
*On target:* recognition of tumor-antigen on normal tissues
*Off target:* cross-recognition against molecules expressed on normal tissues.




***CAR redirected T lymphocytes***

**Antigen binding**:extracellular only

**Ag recognition:** HLA unrestricted

**Associated costimulatory molecules:**
1st generation CD3 ζ chain;
2nd generation CD3 ζ + CD28 or 4-1BB;
3rd generation CD3 ζ + CD28 and 4-1BB or OX40

**Potential toxicities**
*On target:* recognition of tumor-antigen on normal tissues

**Transferring of TCR and CAR transgenes into T lymphocytes**

**Main strategies:**
•*Viral approaches*: retroviral and lentiviral vectors; adenovirus vectors (no permanent integration).
•*Non-viral approaches*:electroporation and transposon-based integration
•*RNA transfer*